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Invivo and *Invitro* studies on carboxy terminal domains of leptospiral immunoglobulins of *L. interrogans* Icterohaemorrhagiae

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ABSTRACT

Carboxy terminal domains of leptospiral immunoglobulins of r*Lig*A and r*Lig*B were assessed its hemolytic, cytotoxicity effects invitro and its protective efficacy was studied in hamster models. Experimental recombinant proteins of *Leptospira interrogans* Icterohaemorrhagiae were individually cloned, expressed in prokaryotic system. Purified expressed proteins confirmed by western blot, assessed for its protective efficacy using hamster models. 100% and 93% protection on homologues challenge was observed with r*Lig*A and r*Lig*B with adjuvant respectively which is evident with increased levels of antibody levels of r*Lig*A and r*Lig*B with 98% and 95% specificity. Cytotoxicity was maximum with 14% in Daudi cells with with r*Lig*B and 7% and r*Lig*A. 17% and 4% hemolytic activity with r*Lig*A, and r*Lig*B protein and 11% and 10% in pH-7.0 respectively. *E. coli* (harboring pET15b) membrane fraction used as placebo neither exerted cytotoxicity nor hemolysis was used to compare the leptospiral proteins. Leptospiral immunoglobulins showed least cytotoxicity and hemolytic activity with increased homologues protection in experimental animals

Keywords: *Leptospira recombinant protein, Leptospiral immunoglobulins, immune response, challenge studies, cytotoxic and hemolytic activity.*

INTRODUCTION

Leptospiral bacterins, used as vaccines over canines provide homologues protection with varied clinical signs (leptospiremia with renal shedding) and less effective in heterologous protection (Andre-Fontaine *et al.,* 2003). Antigens expressed during leptospiral infection such as lipopolysaccharides, *Lip*L41, *Lip*L32, *Lip*L21 (Isogai *et al.,* 1986), outer membrane glycoprotein, OmpL1 (Alves *et al.,* 1992), and *in vivo* conditions (Immunoglobulin like proteins, *Lig*A, *Lig*B and *Lig*C) plays an imperative role in design and development of vaccines (Naiman *et al.,* 2002).

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Virulent factors that are produced during in vivo conditions increases the pathogenesis, impetus as critical markers for safe vaccine development. In contrast, certain leptospiral virulent factors include phospholipases, sphingomyelinases (Bernheimer and Bey, 1986) and immunoglobulins, may exert protective efficiency in animal models through humoral immunity that leaves severe lesions in tissues (Raghavan *et al.,* 2002). Although, these virulent markers elicits elevated antibody response that limits with cytotoxic and haemolytic activities to the host (Lee *et al.,* 2002). These elevated antibodies are in response to the antigens that are induced due to physiological and osmotic system of the host (Choy *et al.,* 2007).

Leptospiral immunoglobulin genes, *Lig*A and *Lig*B are a class of bacterial immunolglobulin (BIg) proteins that contain tandem repeats of domain specific to *Leptospira* spp (Croda *et al.,* 2007) that corresponds to bacterial adhesions, such as intimin and invasion of *E. coli* and *Y. pseudotuberculosis*, respectively, that involves in microbial pathogenesis (Palaniappan *et al.,* 2002 & Matsunga *et al.,* 2003). The leptospiral *Lig* proteins are surface localized, the expression of *Lig* is differential that increases in vivo and alleviates when

cultured. Expression of *Lig* proteins is found in low passage cultures also in culture isolated from infected animals (Palaniappan *et al.,* 2002). *Lig* proteins are considered as pathogenic and virulence index of the *leptospira* which plays a vital role in pathogenesis as its expression is high *in vivo*. Elevated antibody levels from the sera samples of patients recovering from infection of leptospira indicate the expression of *Lig* proteins by infective spirochetes (Matsunga *et al.,* 2003). Whole gene sequence of *Lig*A and *Lig*B genes revealed that, the amino terminal group of *Lig* proteins are conserved whereas the carboxy terminal portion are variable that are specific to *leptospira* (Raghavan *et al.,* 2006). Moreover the *Lig* proteins expression is osmolarity dependent, the presence of *Lig* proteins in host indicates the *Lig* expression is controlled by host osmotic signal which induces the virulence in leptospires. Attenuation studies of *Lig* genes of *Lig*A and *Lig*B revealed that these are surface localized and are associated with virulence, whereas the *Lig*C genes identified in *L. interrogans* serovar Copenhageni and *L. krischneri* serovar grippotyphosa revealed that it is likely to be as pseudogene and neither associated in virulence nor in pathogenesis (Matsunga *et al.,* 2003).

The present study envisages on the comparison of the carboxy terminal domains of leptospiral immunoglobulin's *Lig*A, *Lig*B gene from *L. interrogans* serovar Icterohemorrhagiae serogroup RGA for its protective efficacy, cytotoxic and hemolytic activities. Experimental leptospiral genes were cloned and expressed in prokaryotic (*E. coli)* host. The expressed proteins were purified by affinity column chromatography and were assessed for its protective potential in hamster models by challenge studies, as well for its cytotoxic and hemolytic activities.

Materials and Methods

Cultivation of *Leptospira:*

L. interrogans serogroup Icterohaemorrhagiae serovar RGA type reference culture was aseptically inoculated on to liquid EMJH medium supplemented with *Leptospira* supplement (BD Biosciences, USA) and maintained at 30 °C under aerobic conditions and collected at a density of approximately 5 $\times 10^8$ bacteria per mL by direct counting of motile bacteria under dark field microscopy in our laboratory. The virulence of the *L. interrogans* was maintained by repeated infection and re-isolation from golden syrian chinese hamsters.

Genomic DNA Isolation and PCR

Genomic DNA was isolated (STET Method - Bartlett and Stirling, 2004) from *L. interrogans* serovar *icterohaemorrhagiae* strain RGA and amplification of *Lig*A and *Lig*B genes were performed by PCR (ABi 9700 thermal cycler) using gene specific primers. The gene primers were designed with *Nde*I and *Xho*I recognition sites (***Lig*A FP-** 5' GGG TTT CAT ATG GCT GGC AAA AGA GGC - 3'; ***Lig* A FP- *5***'-GGG CTC GAG GTC TCT CCA GTT TTA CC -3'; ***Lig*B FP** 5'-CAC AAG CTT CAT ATG TAC GCA TAT T-3' ***Lig*B RP** 5'- TTA CTC GAG CTT ATA CGA ATT AC -3'). PCR amplicons were analyzed in 1.5% agarose gel electrophoresis. The PCR amplicons were purified using Qiagen PCR product purification kit. Then, purified products were sequenced and analyzed using BLASTn analysis. The nucleotide sequences were edited through BIO EDIT software and aligned through CLUSTALX. The aligned sequence of *L. interrogans* Icterohaemorrhagiae RGA was submitted to NCBI, Genbank.

Cloning and expression of r*Lig*A and r*Lig*B gene from Icterohaemorrhagiae:

The vector (pET15b) plasmid was extracted using Qiagen miniprep kit and the plasmid was analyzed in 1% agarose gel electrophoresis. The isolated pET15b plasmid and the purified *Lig*A and *Lig*B amplicons were digested with *Nde*I and *Xho* I restriction enzymes (New England BioLabs) at 37 ºC for 3 h and purified using Qiagen gel extraction kit. The digested pET15bp plasmid and PCR products were *Lig*ated individually at 16 ºC for overnight. The *Lig*ated products were transformed into *E. coli* DH5α cells. The pET15b and recombinant gene constructs were extracted from *E. coli* DH5α cells and were transformed into *E. coli* BL21 (DE3) codon plus RP cells. Recombinant colonies were screened using colony PCR and digestion of recombinant plasmid with restriction enzyme digestion (*Nco*I and *Xho*I) to check the insert release *E coli* BL21 (DE3) cells harboring recombinant plasmids were grown in LB medium supplemented with ampicillin at 50mg/L at 37 ºC for overnight. *E. coli* cells harboring the recombinant gene constructs were individually induced with 1mM of IPTG *E. coli* BL21 DE3 harboring the pET15b vector alone was induced simultaneously for 16 hrs as negative control. The protein expression was carried out at 28ºC for optimal induction. The induced and uninduced *E. coli* cells were lysed by heat denaturation and resolved in 12% SDS-PAGE.

Purification and confirmation of r*Lig*A and r*Lig*B protein:

The recombinant proteins were purified using Ni^{2+}-NTA affinity column chromatography as per the manufacturer's instructions (Invitrogen, USA). The purified protein fractions collected and the eluted protein were analyzed by 12% SDS-PAGE and analyzed through coomassie brilliant stain R-250 (Merck, USA) staining. The SDS-PAGE analyzed purified recombinant proteins were transferred on to Polyvinylidene fluoride (PVDF) membrane (Whatman, UK). Western blot was carried out using BioRad western blotting system as per the manufacturer's protocol. The PVDF membrane was then treated with hyper-immune serum (1:200) raised against virulent leptospires in hamster models to assess the specificity of the expressed recombinant proteins.

Latex agglutination test (LAT):

The whole cell antigen preparation and the recombinant antigen of r*Lig*A and r*Lig*B were used for coating the carboxylate modified polysterne latex beads (size 0.8µm, Sigma Aldrich, USA) and screened through recombinant antigen induced sera samples. The LAT assay was carried out as per the previously reported procedure (Senthilkumar *et al.,* 2008). The test results were read within 2 min. The test score was positive if agglutination occurred, indicated by the formation of fine granular particles, which tend to settle at the edge of the droplet and scored negative if the suspension remains homogenous. The positive samples were graded as +1 to +3.

Cytotoxicity of r*Lig*A and r*Lig*B against Mammalian cell culture:

In-vitro cytotoxicity assay was carried out for r*Lig*A and r*Lig*B protein using mammalian cell lines. HEK-293 (Human embryonic kidney cell line), Vero (African green monkey kidney cell line), HepG2 (Human liver carcinoma cell line), Jurkat E6.1(Human T lymphoblast cells) and Daudi cells (Human B lymphoblast cells) were obtained from National Centre for Cell Sciences, Pune were maintained in our lab. Adherent HEK 293, Hep G2 and Vero cells were maintained to confluent culture in Dulbecco's modified Eagle's culture medium (DMEM) (Sigma Aldrich, USA) supplemented with 10% FBS (fetal bovine serum) and Jurkat E6.1 and Daudi cells in Roswell park memorial institute medium (RPMI) (Sigma Aldrich, USA) supplemented with 20% FBS were placed on to 96-well plate (Corning, USA) and incubated at 37°C in 5% CO_2 for 12 to 15 h. Both the medium were buffered with sodium bicarbonate and supplemented with 100µg of Penicillin and 100µg of Streptomycin sulphate (Sigma Aldrich, USA). A confluent monolayer and suspension of cells with a viability of 95-98% was trypsinized (adherent cells alone),washed twice with phosphate-buffered saline (Sigma, USA) and added with serially diluted recombinant proteins (r*Lig*A and r*Lig*B) from 5, 10, 25, 50, 75, 100, 150 and 200 µg/mL. The treated cells were incubated for 10 h at 37°Cin 5% CO_2. The plates were centrifuged at 800 x g for 5 min and 100 µL of supernatant was aspirated and mixed with substrate solution on to another sterile 96 well plate and incubated for 30 min at room temperature. The cytotoxicity was denoted by the lactate dehydrogenase (LDH) release in treated cells with recombinant proteins were measured (Del Real *et al.,* 1989) using a CytoTox96 cytotoxicity kit (Promega, USA). 50 µL of stop solution was added and measured at 490 nm. Phospholipase C from *Clostridium perfringens* (Sigma, USA) (50 µg/ml) and membrane fraction of *E. coli* (pET15b) (Lee *et al.,* 2002) were used as control, respectively.

Where xsample was absorbance at 490 nm of supernatant obtained from treated cells and xbackground were the absorbance of supernatant obtained from un-treated cells and xtotal is from supernatant of cells lysed with lytic buffer, respectively. The assay was carried out on triplicates, repeated three times and graph was plotted with the mean values.

Cytotoxicity was calculated by using the formula:

$$\text{Percentage of Cytotoxicity} = 100 \times \frac{(\text{xsample} - \text{xbackground})}{(\text{xtotal} - \text{xbackground})}$$

Hemolytic activity of r*Lig*A and r*Lig*B:

r*Lig*A and r*Lig*B protein was assayed for hemolytic activity as per the reported procedure (Alves *et al.,* 1992). Recombinant proteins at different concentrations were added to the 10% suspension of rabbit erythrocytes was prepared with 10mM Tris-Cl (pH 7.5) and 100mM NaCl. Complete lysis of the suspension was assessed by adding it to sterile distilled water.

Immunization of hamsters with purified recombinant proteins:

Hamsters of 4 weeks old were grouped into 5 groups with each group and sub group (III, IV, V) consisted of 5 animals.

Group I :	Control with PBS
Group II:	50 µg of membrane fractions of pET15B harboring *E. coli* cells.
	50 µg of purified and dialysed r*Lig*A protein
Group III :	50µg of r*Lig*A protein with adjuvant (Aluminum hydroxide)
	50 µg of purified and dialysed r*Lig*B protein
Group IV :	50µg of r*Lig*B protein with adjuvant

The groups were subcutaneously immunized with recombinant proteins as per the given schedule. Hamsters maintained in the animal house were immunized from day 0 and subsequently boosted on day 21 for recombinant protein and day 42. Hamsters were bled on day 0 (pre vaccination) 21 and 42 directly from saphenous vein from all the groups and the serum was collected (Haake *et al.,* 1999). The experiments were conducted in triplicates and the tissues were collected aseptically for culture and histopathological analysis.

Determination of Lethal dose (LD_{50}):

Virulent *L. interrogans* Icterohaemorrhagiae RGA obtained from infected hamsters were passaged twice in EMJH medium was serially diluted aseptically to 10 fold (10^9-10^5) were injected intraperitoneally in to 8-9 weeks old hamsters. The $LD_{50}$was calculated (Reed and Munch, 1938) and was devised as 10^6 dilutions. Ethical clearance was obtained from 20th Institutional Animal Ethical Committee held at Madras Veterinary College.

Challenge Experiment:

The control groups were immunized with PBS and the placebo groups received membrane fraction of pET15 in PBS. On day 42, the hamsters groups were challenged with approximately 1 LD_{50} (6 x 10^3) of *L.*

interrogans serovar icterohaemorrhagiae RGA injected intraperitoneally. Hamsters were bled before the immunization (day 0), 1 day before the challenge (day 41) and at the time of killing (day 71). Antibody titers were determined as described above. After challenge (on day 71), the extant hamsters were sacrificed and the tissues (liver, kidney and blood) were collected aseptically from different vaccinated groups and cultured to assess the presence of *L. interrogans*. Liver and kidneys were homogenized aseptically in 0.5mL sterile EMJH medium and transferred to 20mL EMJH medium and maintained at 30 ºC for 4 weeks for growth. The growth of *Leptospira interrogans* was measured using dark field microscopy.

Enzyme linked immunosorbent assay with recombinant antigens:

Sera samples collected on days 0, 21, 42 and 71 days from hamsters receiving r*Lig*A and r*Lig*B with adjuvants, were assayed for the presence of Specific IgG using ELISA assay with r*Lig*A and r*Lig*B protein preparation of *L. interrogans*.

Statistical analysis:

The data presented in the study were expressed as mean±SD. The differences between groups were evaluated by one-way analysis of variance (ANOVA), and multiple comparisons were carried out by Dunnett's test of Prism GraphPad version 5.0. The values were considered statistically significant where $P<0.05$.

RESULTS & DISCUSSION

PCR amplification and sequence analysis of *Lig*A and *Lig*B genes:

In this study, the *Lig*A and *Lig*B gene from *L. interrogans* Icterohaemorrhagiae RGA was amplified. The PCR conditions were optimized and the expected amplicons size of *Lig*A (691 bp) and *Lig*B (2086bp) were amplified at 52 ºC and 56 ºC of annealing temperature was observed in 1.5% agarose gel electrophoresis (Fig.1). The amplified and purified gene products were sequenced (Genetic analyzer 3130 (ABi systems)). BLAST analysis of the sequenced nucleotide revealed 98% and 97% homology respectively with other *Leptospira* sequences from the NCBI database respectively. The nucleotide sequence edited through BIO-EDIT and aligned by CLUSTALW was submitted to NCBI, Genbank.. **(Accesssion Number: GQ477369 (*Lig*A); GU552679 (*Lig*B))**

Cloning, expression and purification of recombinant protein

A precise band of 691bp (*Lig*A) and 2086bp (*Lig*B) were observed in colony PCR, whereas two bands with respective base pair size of gene insert and 5708bp plasmid vector (pET15b) were observed in insert release. The optimal expression of gene constructs was standardized and obtained at 1.0mM concentration of IPTG at 28 ºC and the expression of gene was noticed from the first hour of induction till fifth hour of post induction at a molecular weight 24kDa and 74kDa in comparison to uninduced controls and tends to decrease after fifth hour in both the gene constructs was observed in expression. The purified recombinant proteins of 24kDa and 74kDa were observed in 12% SDS-PAGE was observed by CBB-R250 Staining (Fig.2). The induced, eluted fractions of recombinant proteins were confirmed using western blotting and were observed as precise band of respective size on the PVDF membrane when developed using hyper immune serum raised in hamsters (Fig.3) and the specificity of the antibodies against the recombinant proteins were confirmed by latex agglutination test.

Figure-1: Agarose Gel Electrophoresis of Amplified gene products

Lane 1: 10Kb Ladder; Lane 2: *Lig*A gene amplicons (691 bp); Lane 3: *Lig*B gene amplicon (2000b

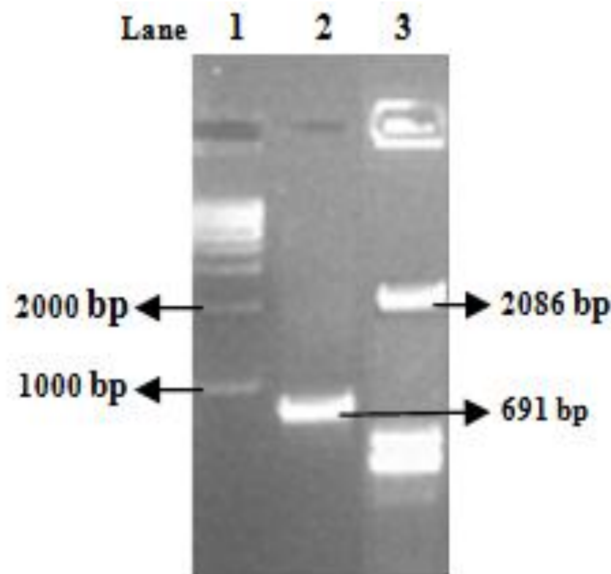
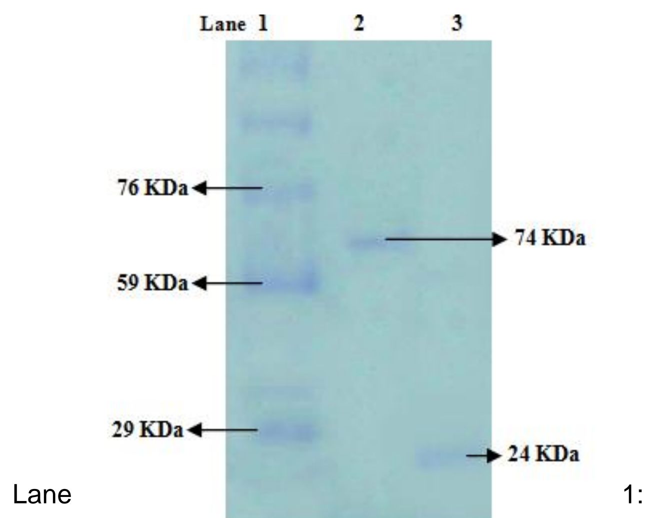


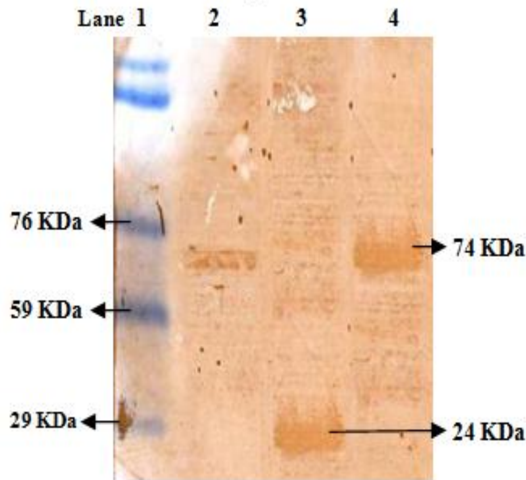
Figure-2: His-Tag Ni^{2+}-NTA affinity purified protein PAGE analysis r*Lig*A and r*Lig*B proteins



Lane 1:

Protein Marker (Medium range)
Lane 2: Internal control (LipL41)
Lane 3: r*Lig*B (74 KDa)
Lane 4: r*Lig*A (24 KDa)

Fig 3: Western blot analysis of recombinant protein

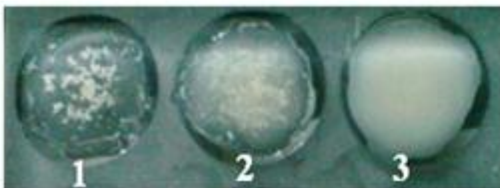


Lane 1: Pre stained protein marker (Medium range)
Lane 3: r*Lig*A (24 KDa)
Lane 4: r*Lig*B (74 KDa)

Latex Agglutination Test:

The agglutination with the recombinant proteins (r*Lig*A and r*Lig*B) and the whole cell samples were similar and were graded as +3 for the purified recombinant protein samples which reacted readily within 1 min. and +2 in whole cell preparation of *L. interrogans* which reacted upon continuous mixing and agglutinated within 2 min. Results were negative (no agglutination) with uninduced sera samples obtained from control hamsters injected with distilled water used as mock (Figure-4).

Figure-4. Latex Agglutination Test

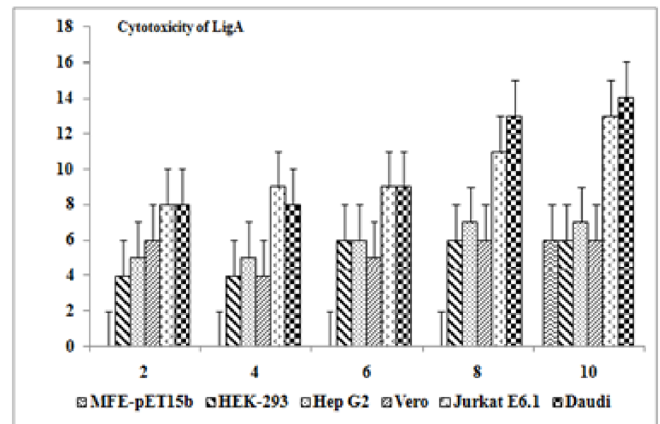


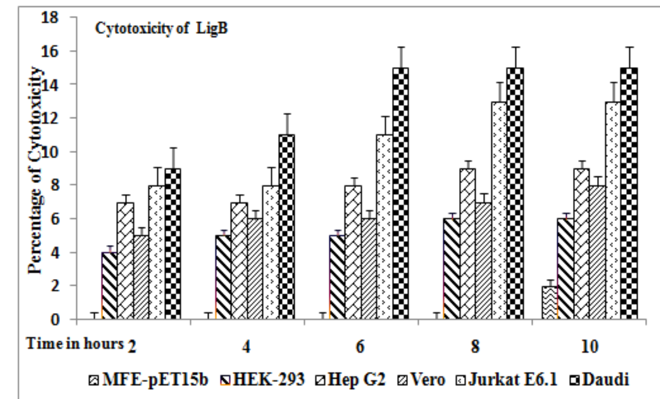
1. Latex beads coated with r*Lig*A protein assayed with antibody received from r*Lig*A
2. Latex beads coated with r*Lig*B protein assayed with antibody raised against r*Lig*B protein
3. Latex beads coated with whole cell antigen assayed with PBS

Cytotoxic and hemolytic activity of r*Lig*A and r*Lig*B protein.

Recombinant proteins with concentration ranging from 5-100 *µg/mL* showed the LDH release is dose dependent and attained maximum at of 300 *µg/mL* for r*Lig*A and r*Lig*B. Cytotoxicity induced by r*Lig*A and r*Lig*B protein was less when compared with Phospholipase C 50 *µg/mL* from *C. perfringens* (positive control) which induced 100% cytotoxicity. *Leptospiral* r*LigA and rLigB* expressed and purified, had meagre effect on the LDH release. r*Lig*A at 100µg/mL showed meagre cytotoxicity against all the mammalian cells tested (r*Lig*A (HEK-293 (6%), Hep G2 (6%), Vero (7%), Jurkat E6.1 (8%) and Daudi (7%)) (Fig.6) and r*Lig*B (HEK-293 (6%), Hep G2 (7%), Vero (6%), Jurkat E6.1 (13%) and Daudi (14%)).

Figure-5 & 6: Cytotoxicity of recombinant leptospiral proteins in different cell lines r*Lig*A and r*Lig*B



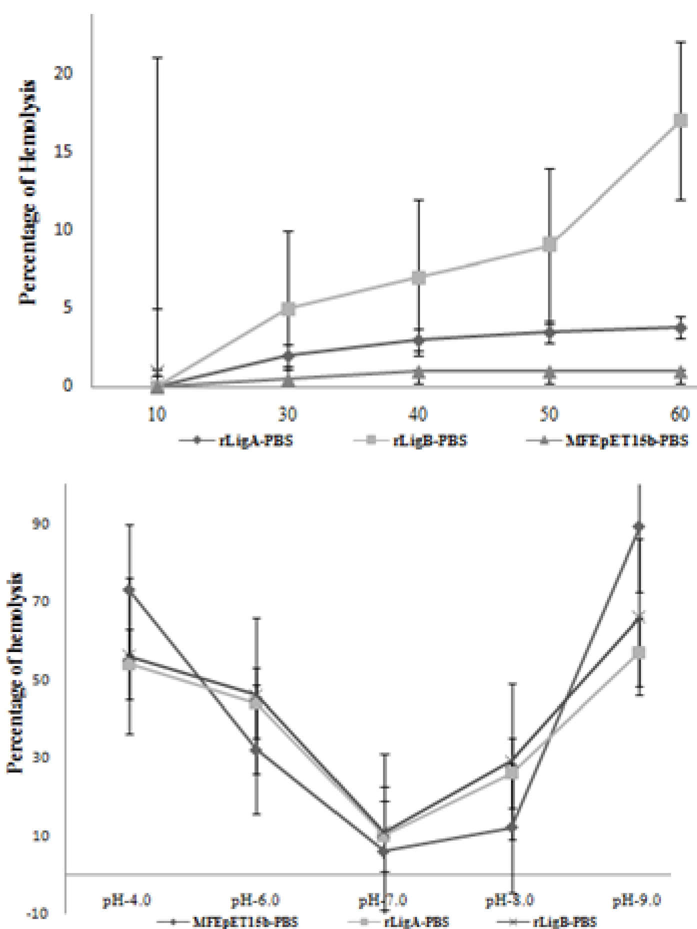


MFE-pET15b - Membrane fractions of *E. coli* harboring pET-15b
HEK-293 - Human embryonic kidney cell lines
HepG2 -Human liver carcinoma cell line
Vero -African green monkey kidney cell line
Jurkat E6.1 -Human T lymphoblast cells
Daudi -Human B lymphoblast cells

The cytotoxicty was 8% - 10% with both undiluted r*Lig*A and r*Lig*B cells. Inverted Phase contrast microscope examination of the tested cells after incubation with Phospholipase-C revealed morphological changes with shrunken and loss in confluence in the initial hour of treatment and found disintegrated as the incubation prolonged whereas the r*Lig*A and r*Lig*B showed similar morphological changes

during the initial stages and recovered with least effect of LDH release. Further, No remarkable changes were found with respect the control cell lines and the cells treated with membrane fractions of *E. coli* harboring pET15b. Hemolytic activity was rising as incubation prolongs with recombinant proteins for rabbit erythrocytes and reached up to 4% and 9% with r*Lig*A and r*Lig*B as well it was 6% and 10% when incubated at pH-7.0 for 60 min respectively (Fig. 8 & 9). Membrane fraction of *E. coli* harboring pET15b showed no hemolytic activity nor cytotoxicity was observed.

Figure-7. Hemolytic activity of r*Lig*A and r*Lig*B in rabbit erythrocytes & Fig.8: Hemolytic activity of r*Lig*A and r*Lig*B at different pH



r*Lig*A-PBS- Purified r*Lig*A protein in phosphate buffered saline
r*Lig*B-PBS- Purified r*Lig*B protein in phosphate buffered saline
MFE-pET15b -PBS - Membrane fractions of *E. coli* Harboring pET-15b in phosphate buffered saline

Antibody response to recombinant antigens:

Sera samples collected on 0, 21, 41 and 71 days from all the groups of hamsters were analyzed by ELISA. Elicited antibody levels were found significant in hamsters receiving, especially in r*Lig*A when compared with r*Lig*B proteins and with adjuvant in comparison to controls treated with PBS (Table-1). On the other hand, antibody levels were not statistically significant in hamsters injected with membrane fractions of pET15b antigen when compared with recombinant proteins response. Increase in antibody levels in hamster receiving r*Lig*A and r*Lig*B proteins indicates the effective surface binding of these virulent factors in turn elevates protective immunity.

Challenge studies and *Leptospira* culture from tissues:

The protective efficacy of r*Lig*A and r*Lig*B protein is presented in Table-2. Hamsters receiving r*Lig*A and r*Lig*B proteins with adjuvant showed 100% and 93% survival whereas, 20% in membrane fractions of pET15b and 6% in controls receiving PBS was observed. Histopathological examination of controls injected with PBS revealed the evidence of acute interstitial nephritis inferred through interstitial inflammation with lymphocyte, macrophage and plasma cells infiltration in the kidney tissue. Mild to severe steatosis with cholestasis and necrosis were observed. This in accordance with severe lesions that was observed in hamster group vaccinated with membrane fractions of pET15b. However, the symptoms were least in the case of animals in the groups treated with r*Lig*B and no demonstrable lesions with r*Lig*A and adjuvant were observed (Results not shown). Tissues from all the control challenged animals showed growth in EMJH medium and presence of organism was observed on dark field microscopic examination. In contrast, tissues from survived animals vaccinated with r*Lig*A showed no

Table 1: ELISA - Antibody titre levels of hamsters vaccinated with r*Lig*A, r*Lig*B and with adjuvant

	0th day	21st day	42nd day	Challenge
Control-PBS^{a}	0.0435 ± 0.001	0.0437 ± 0.001	0.043 ± 0.0009	0.483 ± 0.0007
MFE-pET15 ^{ab}	0.0436 ± 0.002	0.0526 ± 0.004 ^{ns}	0.161 ± 0.007 ^{ns}	0.502 ± 0.006 ^{ns}
r*Lig*A protein	0.0432 ± 0.001	0.634 ± 0.01 ^{a*}	1.876 ± 0.03^{a*}	2.522 ± 0.002^{a*}
r*Lig*A + adjuvant	0.0434 ± 0.001	0.667 ± 0.02 ^{a*}	1.976 ± 0.001^{a,ab**}	2.693 ± 0.004 ^{a,ab**}
r*Lig*B protein	0.0430 ± 0.001	0.520 ± 0.003 ^{a**}	1.676 ± 0.002 ^{a,ab**}	2.142 ± 0.002 ^{a,ab**}
r*Lig*B + adjuvant	0.0432 ± 0.002	0.547 ± 0.01^{a*}	1.962 ± 0.001 ^{a,ab**}	2.360 ± 0.002 ^{a,ab**}

The values are mean±SD, ^{a} Samples compared with Control-PBS; ^{ab} Samples compared with rMFE-pET15b;
* Significant p<0.05; **Significant p<0.01; ^{ns} Not Significant.

Table 2: Protective efficiency of r*Lig*A and r*Lig*B in hamster models

Protective efficiency of r*Lig*A & r*Lig*B in hamster models				
Groups	**No. (%) of Surviving animals / groups[a]**			
	Expt1	**Expt2**	**Expt3**	**Total**
Control	1/5 (20)	1/5 (20)	0/5 (0)	2/15 (13)
MFE-pET15b	1/5 (20)	0/5 (0)	2/5 (40)	3/15 (20)
Significance	P<0.001	NS	P<0.003	NS
r*Lig*A protein	5/5 (100)	4/5 (80)	5/5 (100)	14/15 (93)
r*Lig*A + adjuvant	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
Significance	P<0.001	P<0.001	P<0.001	P<0.001
r*Lig*B protein	4/5 (80)	4/5 (80)	5/5 (80)	13/15 (86)
r*Lig*B + adjuvant	4/5 (80)	5/5 (100)	5/5 (100)	14/15 (93)
Significance	P<0.001	P<0.002	P<0.002	P<0.003

[a]Number of surviving animals after challenged. Statistical analysis was performed using Student's T test-two independent means (Significance, P<0.05); NS, Not significant

growth whereas r*Lig*B showed least to no growth were noticed in triplicate samples (1x10 counts / ml using petroff-hauser counting chamber under Dark field microscopy).

Discussion

Pathogenicity of the bacteria is attributed by the adhesion of the microorganism to the host and its infection duration is critical for the colonization of bacteria or its removal from the host. (Patti *et al.,*1994). *Lig* expression is enhanced by the binding of the spirochete with the host extracellular matrix proteins like fibronectin, collagen and laminin (Ito *et al.,* 1987). Further, difference or increase in the osmotic condition derived from host or in other environment induces the virulence of the spirochete, *Leptospira*. (Matsunga *et al,* 2005) This in accordance, this study anticipates the expression of *Lig* proteins were found significant in leptospires, obtained from the hamster borne cultures and the positive expression of the virulent factors and immunoglobulins are host inducible. Moreover, our findings were corroborated with the available reports suggesting that the loss in virulence or the proteins involves in infection tends to diminish when it is passaged extensively and maintained in artificially buffered medium The poor expression of *Lig* proteins or its lack of detection in vitro grown cultures of leptospires indicates that these proteins are unstable at in-vitro conditions and its expression drops down over a period of subculture. (Palaniappan *et al.,* 2002; Matsunga *et al.,* 2003). The re-occurrence of the expression of *Lig* proteins (*Lig*A and *Lig*B) in pathogenic leptospires and its non-expression in non-pathogenic leptospires denotes that these immunoglobulins are important and effective virulent factors that could be used for serodiagnosis. (Palaniappan *et al.,* 2004).

The expression of complete ORF of *Lig*A in *E. coli* imparted low levels of protein synthesis due to its high toxicity (Palaniappan *et al.,* 2002). With reference to this report, in our study we had targeted the conserved domains of carboxy terminal with high tandem repeats as our study of interest in order to achieve moderate to amplified expression. The *Lig*A protein production was achieved as 1.2 mg/L with the use of *E. coli* BL21 DE3 cells. Similarly, the *Lig*A expression was achieved at an increased fold when it was expressed in *Pichia pastoris* which showed no effect on toxicity while expression (Hartwig *et al.,* 2010). Complete ORF of r*Lig*A induced protective efficacy was found to be 100% when tested with 50 µg of r*Lig*A over hamsters, but with moderate to severe lesions in histopathology of kidney was reported (Palaniappan et al, 2006). Whereas, in our study, no such severe lesions were noticed in kidney and liver tissues when vaccinated with 50 µg of carboxy terminal portion of r*Lig*A from *L. interrogans* serovar Ictero-haemorrhagiae. Our results were in accordance with previous report on r*Lig*A with three domain region induced protection from lethal infection by *L. interrogans* (Mariana *et al.,* 2011). Targeted mutagenesis in respect of *Lig* B gene inferred with no significant effect on the virulence of the leptospires was reported (Croda *et al.,* 2008). Although the *Lig*B imparts homologues protection when challenged with the virulent leptospires, the previously reported study suggests that even at the extant of disruption of *Lig*B, the virulence remained unaltered which could be attributed to the leptospires possibly when *Lig*A expression is functional. Cytotoxicity and haemolytic assay of *Lig* proteins inferred that, these proteins in comparsion with the hemolysins were predominantly significant with least lethal effect to the cells when treated. Even though, the elicited antibody levels and protective efficacy were comparable with the animals vaccinated with the membrane fractions of *E. coli* harbouring pET15b, the aberrative lesion which remained prominent which is indistinct with *Lig* proteins.

ELISA of purified r*Lig*A and r*Lig*B protein showed 96.5%, 94.2% sensitivity and 98.0%, 95% specificity respectively. These results were corroborated with the

previously reported studies of the antibody specificity and sensitivity of rGroEL protein from *L. interrogans* serovar Autumnalis. (Natarajaseenivasan *et al.,* 2011). With respect to r*Lig*A and r*Lig*B the increase in concentration had no significant effect as it had achieved the protective efficacy in 50µg. It is evident through the elevated levels of antibody in the surviving animals immunized with r*Lig*A and r*Lig*B and the least lesions observed in histopathology observed after challenging. The pathogenic leptospires expressing these virulent factors disrupts the cell barriers and enters the blood stream of hosts resulting in systemic spread. The damage to the cell membrane results in hemorrhage, hepatic damage and renal failure leading to severe leptospirosis (Bulach *et al.,* 2000). Hemolysins from *L. interrogans* has lytic action on red blood cells (Pinkney *et al.,* 1989), cytotoxicity (Goldstein *et al.,* 1990) has been studied already on different serovars.

Acute interstitial nephritis, extravasated blood vessels with interstitial inflammatory infiltration composed of lymphocytes, macrophages, plasma cells and eosinophils followed by fatty hepatocytes, choletasis and other hepatic abnormalities were noticed in challenged control and *E. coli* membrane fractions treated hamsters. Whereas the said lesions were less evident or not at demonstrable levels within the groups treated with r*Lig*A and r*Lig*B with adjuvant were observed. Similar pathological conditions were reported in control hamster models with severe chronic and acute degenerative lesions in liver, kidney and lungs when challenged with *L. borgpetersenii* Hardjo (Zuerner *et al.,* 2011). These results suggest that the experimental recombinant proteins mediated diagnostic method would be effective as it is expressed as a virulent factor and are expressed in significant levels. Identification of structural motifs of these experimental proteins of *L. interrogans* possibly helps to understand the proteins that could be used as potential diagnostic and vaccine candidate

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper

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